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Gene Expression from the Imprinted Dio3 Locus Is Associated with Cell Proliferation of Cultured Brown Adipocytes

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Active thyroid hormones are critical for the differentiation and function of brown adipose tissue. However, we have observed high basal and induced levels of type 3 deiodinase (D3), an enzyme that inactivates thyroid hormones and is coded by the imprinted gene *Dio3*, in differentiating brown preadipocytes in primary culture. We find that D3 activity and mRNA expression strongly correlate with the rate of proliferation of undifferentiated precursor cells under various conditions. Furthermore, differentiation of precursor cells to adipocytes is associated with decreased levels of D3 expression, and only very low levels of D3 mRNA are found in mature adipocytes. *Dlk1*, an inhibitor of adipocyte differentiation and a paternally expressed gene located in the same imprinted domain as *Dio3*, displayed changes in expression that parallel those of *Dio3*. In contrast, a 4-kb transcript for *Dio3os*, an antisense gene also located in the same imprinted domain, is markedly up-regulated in differentiated adipocytes. We conclude that D3 expression in differentiating preadipocytes is primarily linked to proliferating cells, whereas *Dio3os* expression is associated with mature adipocytes. Our results suggest that genomic imprinting and gene expression at the *Dlk1/Dio3* imprinted domain may play a role in the regulation of adipocyte proliferation and differentiation. (*Endocrinology* 148: 3968–3976, 2007)

BROWN ADIPOSE TISSUE (BAT) is a highly specialized tissue that is essential for adaptive thermogenesis (1, 2). This function is accomplished in large part by the uncoupling protein (UCP)-1, a mitochondrial membrane protein that is able to uncouple ATP synthesis, thus leading to the dissipation as heat of the energy produced by fatty acid combustion (3). BAT is a very vascular tissue, with endothelial cells being the most abundant (4). This prominent tissue vascularization and the location of BAT near major blood vessels ensures an efficient dissipation of the heat generated in response to adrenergic stimuli (2).

Important physiological functions of BAT are regulated by thyroid hormones (TH). TH are necessary for UCP-1 expression and thus BAT thermogenic function (5–11). In this tissue, the biologically active thyroid hormone, T_3, is derived in part locally from the prohormone tetraiodothyronine, T_4, by the action of the type 2 iodothyronine deiodinase (D2) (12–14). The importance of TH metabolism and activation for BAT function is underscored by two observations: 1) BAT D2 is markedly induced after adrenergic stimulation or cold exposure (12, 13) and 2) D2 knockout mice exhibit significant deficits in BAT thermogenesis (6, 15).

Using a primary cell culture model of brown adipocytes differentiating *in vitro* from precursor cells isolated from the BAT vascular stroma, we have shown the importance and regulatory role of TH in BAT (16–22). BAT precursor cells represent a small fraction of the BAT cell population and, under specific culture conditions, briefly proliferate, reach confluence, and differentiate into mature brown adipocytes (23, 24). In this model system, T_3 facilitates adipocyte differentiation because it up-regulates the expression of important lipogenic markers such as malic enzyme and spot 14 (S14) (16, 19). We and others have also shown that T_3 is required for the adrenergic stimulation of D2 (17) and UCP-1 (21, 25).

Interestingly, we found that cultures of differentiating brown preadipocytes express significant amounts of type 3 deiodinase (D3), another enzyme from the deiodinase family that, in contrast to D2, is an inactivating enzyme in that it converts both T_3 and T_4 into inactive metabolites (26, 27). In this cell culture model, D3 is markedly induced by various growth factors (28, 29). However, very little D3 is expressed in BAT *in vivo*, so the physiological significance of D3 in this cell culture model and in BAT remains unclear.

D3 is highly expressed in developmental tissues (30, 31) and coded by an imprinted gene (*Dio3*) that is preferentially expressed from the allele inherited from the father (32, 33). The mouse *Dio3* is located within an imprinted domain in the distal portion of chromosome 12 (34) and features a noncoding, partially overlapping antisense gene (termed *Dio3os*) that is transcribed from the opposite DNA strand (32, 35). Interestingly, *Dlk1* is another imprinted gene in this domain

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Abbreviations: aFGF, Acidic fibroblast growth factor; BAT, brown adipose tissue; bFGF, basic fibroblast growth factor; D2, type 2 iodothyronine deiodinase; D3, type 3 deiodinase; DTT, dithiothreitol; EGF, epidermal growth factor; LPL, lipoprotein lipase; NCS, newborn calf serum; NE, norepinephrine; PTU, propylthiouracil; S14, spot 14; SDS, sodium dodecyl sulfate; SSC, saline sodium citrate; TH, thyroid hormones; UCP, uncoupling protein.

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that is also expressed from the paternal allele (36, 37). *Dlk1* was identified independently by several investigators. One of these reports described Dlk1 in preadipocytes (named in this paper as preadipocyte factor 1) and suggested it played a role as a potent inhibitor of adipocyte differentiation (38, 39).

Here we report that expression of *Dio3* in brown preadipocytes in culture is associated with cell proliferation rather than differentiation and that gene expression from the *Dio3* locus and its imprinted domain is coordinated during brown adipose conversion.

Materials and Methods

Materials

DMEM was from Life Technologies, Inc. (Uxbridge, UK). Newborn calf serum (NCS) was from Flow (Paisley, Scotland, UK). Antibiotics were from the local pharmacy. BSA [BSA in solution at 22% (pH 7.2)] was from Ortho Diagnostic Systems, Johnson & Johnson Co. (Raritan, NJ). Collagenase, bovine insulin, ascorbic acid, guanidinium HCl, 3-*N*-morpholino-propanesulfonic acid, dithiothreitol (DTT), propylthiouracil (PTU), T_3, norepinephrine (NE), and endothelin-1 were from Sigma (St. Louis, MO). Agarose was from FMC Bioproducts (Rockland, ME). Basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF) were from Roche Molecular Biochemicals (Mannheim, Germany). Ion exchange resin AG1-X8 was from Bio-Rad (Richmond, CA). Radiolabeled [α-^{32}P]dCTP (3000 Ci/mmol) was from Amersham International (Buckinghamshire, UK) or MP Biochemicals (Costa Mesa, CA). Formamide was from Fluka (Buchs, Swtizerland) or Merck (Whitehouse Station, NJ), and the oligolabeling system was from Pharmacia, Inc. (Uppsala, Sweden). Nytran membranes were purchased from Renner GmBH (Dannstadt, Germany). Charcoal (Norit A) to prepare hormone-depleted serum was from Amend (New York, NY).

Cultures of brown adipocytes

Precursor cells were obtained from the interscapular brown adipose tissue of 20-d-old rats (Sprague Dawley), isolated according to the method described by Nechad (23), with modifications (17) using collagenase digestion (0.2%) in DMEM+1.5% BSA at 37 C and filtration through 250 μM silk filters. Mature cells were allowed to float and the infranatant was filtrated through 25-μm silk filters and centrifuged. A hypoosmotic shock (to remove red blood cells) was not done. Precursor cells were seeded in 25-cm^2 culture flasks at a density of 1500–2000 cells/cm^2 on d 1 and grown in a standard culture medium for this cell model that consisted in DMEM supplemented with 10% NCS, 3 nM insulin, 10 mM HEPES, 50 IU penicillin, 50 μg streptomycin/ml, and 15 μM ascorbic acid. As determined by RIA, NCS contained 77 nM T_4 and 1.3 nM T_3 before dilution in the culture medium. Culture media were changed on d 1 and every second day thereafter until cells reached confluence or the experiment was performed. Unless otherwise stated, prior experiments of cell stimulation with serum or growth factors, culture media were depleted overnight from 10 to 1–2% of NCS. Precursor cells proliferate actively under these conditions, reach confluence on approximately the fifth day after seeding (40,000–60,000 cells/ cm^2), and then start to differentiate into brown adipocytes.

In addition to the standard culture condition described above, two other special culture conditions were used. The purpose of this special culture medium was to accelerate the differentiation of precursors cells or prevent it by keeping them in a proliferative state. To promote proliferation and prevent differentiation precursor cells were plated at lower density (500 cells/cm^2), medium was changed every day, and cells were passed at d 5 of culture (before reaching confluence) and plated again at the same density and cultured for another 5 d, changing the medium every day. Cells were then treated and used for experiments. To promote differentiation, precursor cells were plated at regular density and medium changed on d 1 of culture and every other day. On the fifth day of culture and onward, a differentiating medium was used. This medium contained 1% of NCS, 4 nM insulin, 2 nM T_3, 50 nM dexamethasone, and 200 nM rosiglitazone. Cells were used at d 10 of culture.

Determination of D3 activity

Cells were scraped, collected in buffer [0.32 sucrose, 10 mM HEPES, 10 mM DTT (pH 7.0)] and homogenized. D3 activity was determined as described (10) by measuring the iodide released after incubation of cell homogenates with 40,000 cpm of inner-ring radiolabeled T_3 (3, 5-^{125}I-T3 from Formula GmbH (Berlin, Germany) with specific activity of 80 μCi/μg) at 37 C during 1 h. Assay final conditions were 20 nM T_3, 20 mM DTT, 80–100 μg protein per 100 μl, 1 mM PTU (pH 7.5) (28). [^{125}I]Iodide was separated from the rest of the reaction products using Dowex 50W × 2 columns as described (17). Three different cultures per treatment were analyzed in duplicate. Protein content was determined by the method of Lowry or using a reagent from Bio-Rad, after precipitation of homogenates with trichloroacetic acid to avoid interference of DTT in the colorimetric reaction. The amount of deiodination in the assay blanks was routinely less than 0.5%. Detection limits were 75–100 fmol/h·mg protein.

D3 activity in interscapular BAT from mice of different age was assayed as above and also by using outer-ring radiolabeled T_3 [3,5,3′-^{125}I-T3; PerkinElmer (Norwalk, CT) with specific activity 3390 μCi/μg]. Tissue was homogenized as described above. In this case, the final assay conditions were 2 nM T_3, 40 mM DTT, 1 mM PTU, and 25–75 μg protein per 50 μl. The radiolabeled T_2 generated was separated from T_3 by paper chromatography (40) and counted. Deiodination in the assay blanks was less than 3% and detection limits were approximately 3 fmol/h·mg protein. Linear deiodination was obtained in the range of protein concentration used, and within-assay variation was less than 10%. The additional use of this differently radiolabeled substrate to measure D3 activity in BAT was due to the increase sensitivity of the assay required to detect low D3 activities, which was not the case in cultured preadipocytes. No significant differences in D3 activity values are obtained when using either radioactive substrate, provided the activity to be measured is above the sensitivity of the particular assay.

Proliferation assays

Preconfluent cells (d 2–3) were subcultured in 24-multiwell tissue culture plates (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) at a density of 8000 cells/cm^2, using 1 ml of culture medium supplemented with 10% NCS. After 6 h the cells were rinsed twice with medium and maintained for 48 h in culture medium supplemented with 2% NCS (time 0). This is the starting point for mitogenic stimulation using serum, hormones and growth factors. The study of cell cycle at time 0 revealed that 95% of cells are in G_0/G_1 phase (quiescent cells). This was determined as follows (41): 1 million cells were centrifuged, suspended in 300 μl ice-cold PBS, and fixed with 900 μl ice-cold ethanol added dropwise with continuous vortexing. After two washes with PBS, the cells were suspended in 850 μl PBS containing 5 μg RNase per milliliter and incubated for 30 min at room temperature. The cells were then stained by adding 125 μl propidium iodide solution [50 μg propidium iodide per milliliter in 50 mM sodium citrate (pH 7.2) and 0.1% Triton X-100] and analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA), by using the CellQuest software from Becton Dickinson Immunocytometry Systems (Mansfield, MA).

[^{3}H]thymidine incorporation assays were performed as described (41). In brief, quiescent cells were stimulated with mitogenic agents at time 0 in the presence of [^{3}H]thymidine (1 μCi/ml). After 40 h of exposure, the medium was discarded and the cells removed from the plate using a trypsin-EDTA solution. Thereafter the contents of each well were harvested onto glass-fiber filters using a cell harvester (Skatron Instruments AS, Lier, Norway) and the radioactivity incorporated into DNA determined using a β-scintillation counter. Alternatively, a cell harvester from Inotech AG (Dottikon, Switzerland) or a MicroBeta from Wallac Oy (Turku, Finland) were used in these studies.

RNA preparation and Northern blot analysis

Total cellular RNA was extracted in guanidinium-HCl as described (16), using ethanol precipitation. The recovery was 60–90 μg total RNA per 25-cm^2 flasks (containing approximately 5 × 10^6 cells). For Northern analysis total RNA (20 μg) was denatured and electrophoresed on a 2.2 M formaldehyde per 1% agarose gel in 1× 3-*N*-morpholino-propanesulfonic acid buffer and transferred to nylon mem-

branes (Nytran) as described (42). A fragment (~1.1 kb) of a rat *Dio3* cDNA clone, corresponding to most of the coding region of the *Dio3*, was used as a probe by labeling with [α-^{32}P]dCTP using random primers (S.A. $> 10^8$ cpm/μg DNA). Filters were hybridized for 20 h at 50 C [40% formamide, 5× saline sodium citrate (SSC), 2 × Denhardt's, 0.1% sodium dodecyl sulfate (SDS)], washed four times in 2× SSC per 0.2% SDS at room temperature for 15 min and then twice in 0.1× SSC per 0.2% SDS at 65 C for 20 min. Autoradiograms were obtained from the filters and quantified by laser computer-assisted densitometry (Molecular Dynamics, Sunnyvale, CA). Some filters were hybridized with cyclophilin as a control to correct for differences between lanes in the amount of RNA. Full-length rat cDNAs for cyclophilin, lipoprotein lipase (LPL), c-*jun*, *Dlk1*, and *S14* were used as probes. For *Dio3os* Northern analysis, a mouse *Dio3os* cDNA fragment (35) (GenBank accession no. AY077457) was used as a probe. This Dio3os cDNA is about 700 bp in length and is primarily complementary to genomic sequences 5′ of the *Dio3* exon, although it has an approximately 20-bp overlap with the 5′ end of the *Dio3* exon (35). Typically, two different cultures were used to isolate two RNA samples per treatment. Two Northern blot analysis were performed using each of the duplicate RNA samples. Representative experiments are shown in the figures.

Results

Basal and induced D3 activity decrease with adipose conversion

Under the standard culture conditions originally described (see *Materials and Methods*), precursor cells isolated from neonatal BAT proliferate *in vitro* and start to differentiate into adipocytes after they reach confluence, which occurs approximately 5 d after plating. When proliferating, precursor cells have a fibroblast-type appearance (Fig. 1, A and B) that rounds up after confluence when they start to accumulate lipid droplets (Fig. 1C). This change from proliferation to differentiation is also evident from the protein and DNA content of the cultures. In preconfluent cells, protein and DNA content rise in unison as cells proliferate, whereas after confluence DNA content remains stabled and protein content continues to increase (Fig. 1D).

Under these culture conditions, basal and induced D3 activities were measured in preconfluent cells (d 4 after plating) and postconfluent cells (d 6 and 7). We observed that basal D3 activity markedly decreases after confluence (d 6) as cells start to differentiate (d 7) (Fig. 2A). D3 activity is stimulated by several factors such as serum (added *de novo*), bFGF, and vasopressin. As shown in Fig. 2A, the stimulation over control values is similar in pre- or postconfluent cells (d 7), but the overall induced D3 activity is decreased in differentiating adipocytes. This pattern of decreasing D3 activity with differentiation is not significantly altered if the data are expressed per DNA content (Fig. 2B), indicating that the decrease in D3 activity is not due to the change in the protein to DNA ratio that is apparent when differentiation initiates (Fig. 1D). Serum stimulation of *Dio3* mRNA is also higher in preconfluent cells (d 4 of culture) than in postconfluent cells (d 7 of culture) (Fig. 2C), again suggesting that differentiation decreases the level of D3 response to certain factors.

Overall, these results demonstrate that the D3 expression is highest in preconfluent, proliferating cells, and thus, the initiation of adipose conversion results in a decrease in basal and stimulated D3 expression.

Serum rapidly increases D3 expression

Serum is widely used to promote cell growth *in vitro*. In confluent cultures of brown preadipocytes, serum treatment rapidly induces D3 expression. After overnight serum depletion, the addition of 10% NCS results in peak values for D3 activity in 3 h with a return to basal levels after 48 h of serum addition (Fig. 3A). At the mRNA levels, Dio3 mRNA induction can be detected after 1.5 h of serum addition and reaches a maximum after 3 h (Fig. 3B, *top*). Induction of early genes is known to mediate some serum effects on cell proliferation. In this regard, we also observed an early induction (45 min) of c-jun in this cell model as a result of serum treatment (Fig. 3B, *bottom*). These findings indicate that induction of D3 is associated with signals that result in cell proliferation.

Correlation between cell proliferation and D3 expression

In previous studies we have shown that various growth factors can regulate D3 expression (28) and cell proliferation (41) in a dose-dependent manner in differentiating brown preadipocytes. We have thus treated preconfluent preadipocytes (d 4 of culture) with multiple growth factors or mitogens and mixes of factors at different doses to analyze how D3 activity and cell proliferation correlate in response to these treatments. A wide range of D3 activities (Fig. 4A) and cell proliferation (Fig. 4B) was achieved by these treatments. Interestingly, both parameters displayed a marked correlation (Fig. 4C) and elevations in D3 activity closely correlate with increases in cell proliferation.

Growth factor regulation of lipoprotein lipase

LPL is an early marker of adipose differentiation, and its expression is detected before confluence in many adipocyte

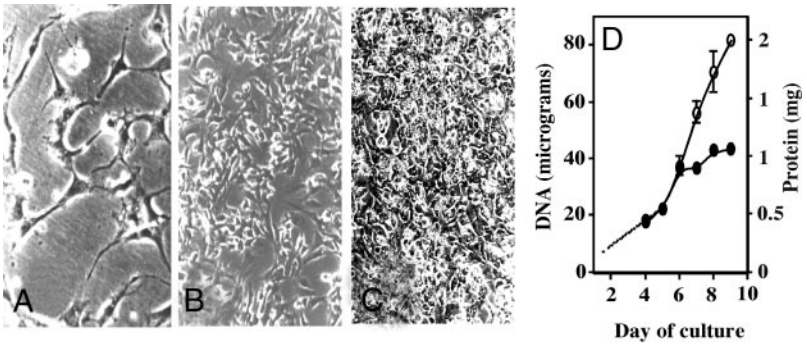

FIG. 1. Morphology, protein, and DNA content of brown preadipocytes differentiating in primary culture. A, Day 3 of culture. B, Day 5 of culture. C, Day 7 of culture. D, DNA (*closed circles*) and protein content (*open circles*) in cultures from 25-cm^2 dishes. Each *point* is the mean ± SD of determinations in a number of culture dishes ranging from 12 to 212.

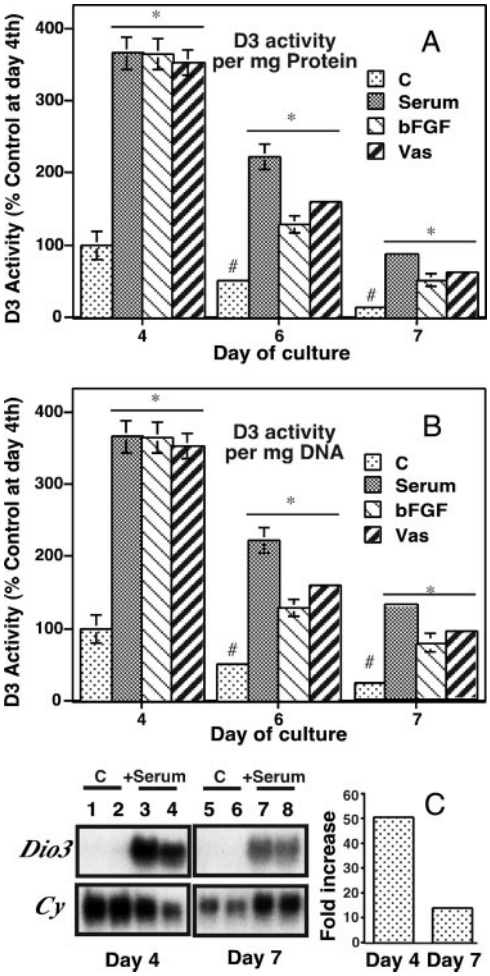

FIG. 2. Basal and stimulated D3 gene expression in brown preadipocytes at different days after seeding. A and B, D3 activity. Basal activity was measured 48 h after changing the medium. Treatments with bFGF (5 ng/ml) and vasopressin (Vas; 20 nM) were done for 7 h and in the presence of 10% NCS for 24 h. Data represent the average ± SD of determinations in six different cultures. Activity is expressed by protein (A) of DNA content (B). *, $P < 0.01$ *vs.* untreated control at the same day; #, $P < 0.01$ *vs.* untreated control at d 4. C, *Dio3* mRNA. Cultures were depleted to 1% serum-containing medium overnight and then some cultures received serum that amounted to 15% of the medium. *Bars* represent the average of the duplicate cultures shown after correction for cyclophilin (Cy). C, Control.

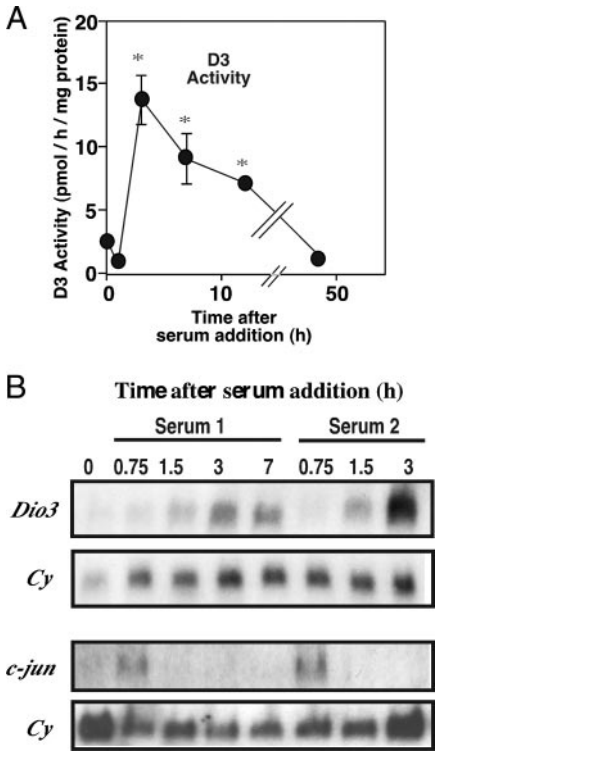

FIG. 3. Time course of induction of D3 activity and *Dio3* and c-*jun* mRNA after serum treatment of brown preadipocytes at d 4 of culture. A, D3 activity. B, Dio3 and c-jun mRNA expression. Twenty micrograms of total RNA were used per lane for Northern analysis. Cy, Cyclophilin. *, $P < 0.0001$ *vs.* time 0.

cell culture models. In our model, expression of LPL in preconfluent cells is down-regulated by different doses of EGF (Fig. 5). Acidic FGF (aFGF), another of the factors that markedly induces D3 activity (28), does not have an effect at a very low dose, but its effect on LPL expression is more pronounced when using a higher but still modest dose (Fig. 5). The finding that factors inducing D3 expression inhibit the expression of an early marker of adipocyte differentiation again suggests that D3 expression is not associated primarily with differentiation.

T_3 is rapidly metabolized in the presence of serum

Our present and previous results showing that serum and growth factors stimulate D3 expression suggested that serum-containing culture media may cause rapid degradation of T_3. To test this hypothesis, we used cultures of confluent preadipocytes and changed their media to a fresh medium containing 10% NCS and added amounts of T_3. The media were sampled 7 and 22 h later and T_3 concentrations were determined by RIA. At physiological concentrations (0.5 and 2 nM), T_3 was rapidly degraded to 60% of the initial values after 7 h (Fig. 6). After 22 h, these values further decreased to only 10% of the initial amount ofT_3. At supraphysiological doses (20 and 50 nM), T_3 concentration decreased to a similar extent after 7 h, despite the far larger initial T_3 values. After 22 h, T_3 values further decreased (40% of initial values), although not as much as with the physiological doses (Fig. 6). These results indicate that, in the presence of serum, T_3 is metabolized very efficiently by the D3 enzyme.

Endothelin I induces D3 activity

Brown fat precursor cells represent a very small fraction of the BAT cell population, and they are mesenchymal cells originated from the vascular stroma of this highly vascular tissue (4, 43). We tested whether endothelin I, a factor highly expressed in endothelial cells, affects D3 expression in brown preadipocytes in culture. Confluent cells treated with different doses of endothelin I for 6 h exhibited a 3-fold induction of D3 activity (Fig. 7). This finding suggests the possibility that precursor cells proliferate as a result of signaling from the endothelial cells of the tissue vascular network.

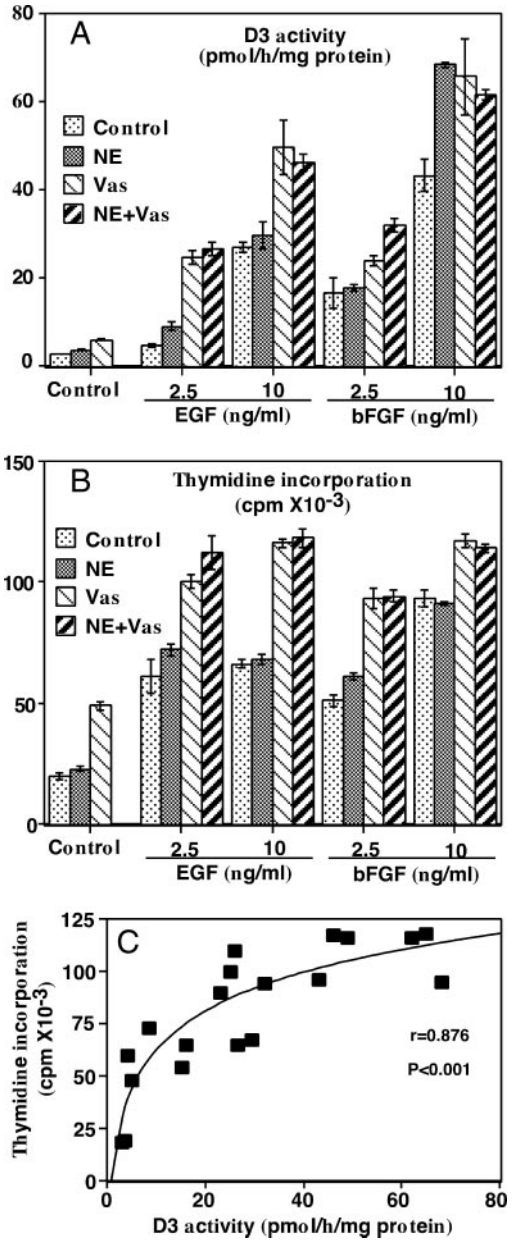

FIG. 4. Relationship between proliferation of brown preadipocytes and D3 activity. A, D3 activity in preconfluent preadipocytes after different treatments. B, Proliferation, measured as thymidine incorporation of preconfluent, quiescent cells after the same treatments. C, Correlation between data from A and B. To measure D3, all treatments were for 7 h (10 ng/ml EGF and bFGF, 1 μM NE, 20 nM Vas). Data represent the average ± SD of determinations in three different culture dishes. Vas, Vasopressin. The equation of the logarithmic fit curve is $y = 26.9\ \mathrm{Ln}(x) - 0.31$.

Gene expression during adipose conversion

To better define the relationship between D3 expression and adipocyte differentiation and analyze gene expression in cells with much larger differences in their state of proliferation/differentiation, we altered the standard culture conditions to promote either cell proliferation or adipocyte conversion (see *Materials and Methods*). To favor proliferation and prevent differentiation, precursor cells isolated from BAT were plated at a low density, and medium containing fetal bovine serum was changed every day. Cells were passed the fifth day of culture before reaching confluence, plated at the same density, and cultured for another 5 d before experiments. To increase the rate of differentiation and obtain fully mature adipocytes, precursor cells were plated at the usual density and treated when confluent with medium containing a low percentage of serum plus T_3, dexamethasone, and rosiglitazone (see *Materials and Methods*). Under both conditions, cells were used after a total of 10 d in culture and gene expression determined using Northern blot analysis.

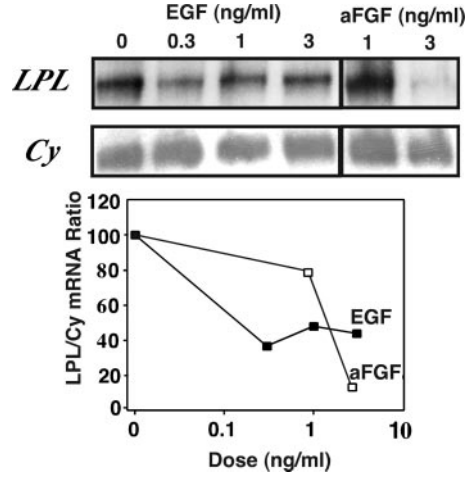

FIG. 5. Effect of different doses of EGF and bFGF on LPL mRNA expression in confluent brown preadipocytes. Cyclophilin (Cy) was used as a control of the RNA loading per well. Cells were exposed to a culture medium depleted of serum (2% NCS) before treatment. Both experiments were performed twice with similar results, and the figure shows data from one of them. All lanes belong to the same blot, although lanes representing aFGF treatments were not contiguous.

Under proliferative culture conditions, cells express basal *Dio3* mRNA (Fig. 8, *top*, lane 1), which can be induced by EGF and bFGF (lanes 2 and 3). However, in differentiated adi-

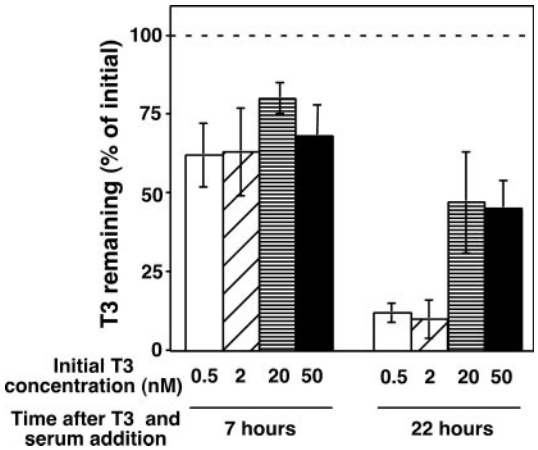

FIG. 6. T_3 disappearance from the culture medium. Confluent preadipocyte cultures were treated with fresh culture medium containing 10% of serum and increasing concentrations of T_3 as indicated. T_3 in the culture media was determined by RIA 7 and 22 h later. The *bars* represent the mean ± SD of values determined in duplicate from three different cultures. The *dotted line* indicates 100% of the initial concentration of T_3.

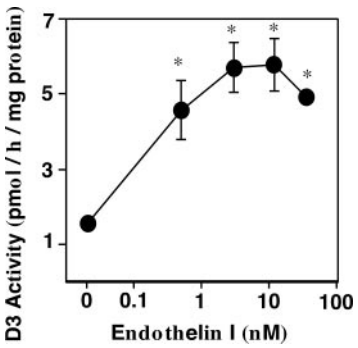

FIG. 7. Dose-response effects of endothelin I on D3 activity in brown preadipocytes. Cells were exposed to a culture medium depleted of serum (2% NCS) before treatment. The data represent the average SD of duplicate determinations in triplicate cultures. *, $P < 0.001$ for of each dose *vs.* untreated control as determined by the Dunnett's test.

pocytes *Dio3* mRNA expression is absent (lane 4) and only barely detectable after bFGF treatment (lane 6). A very similar pattern of expression is observed for *Dlk1*, a gene that is an inhibitor of adipose differentiation and is usually expressed in preadipocytes but not in mature adipocytes (38). Thus, the characterized 1.7-kb *Dlk1* mRNA is also inducible by growth factors in proliferating cells but not in differentiated adipocytes (Fig. 8). In contrast, the expression of S14, a lipogenic marker expressed in adipocytes (44, 45), is absent in proliferating cells but highly expressed in mature adipocytes. This marked difference in the pattern of expression of *Dlk1* and S14 indicates that cells are truly undifferentiated or

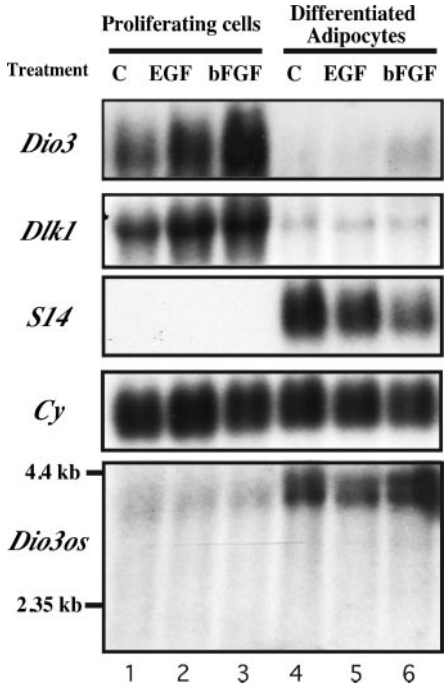

FIG. 8. Gene expression pattern in proliferating precursor cells and fully differentiated brown adipocytes. Cells were cultured, as described in the *Materials and Methods*, in special medium to promote either proliferation or differentiation. Media were then depleted of serum content to 2% the night before and the cells were then treated for 6 h with a 10-ng/ml dose of the indicated growth factors. Blot was autoradiographed for 5 d. C, Untreated cells used as control. Cy, Cyclophilin.

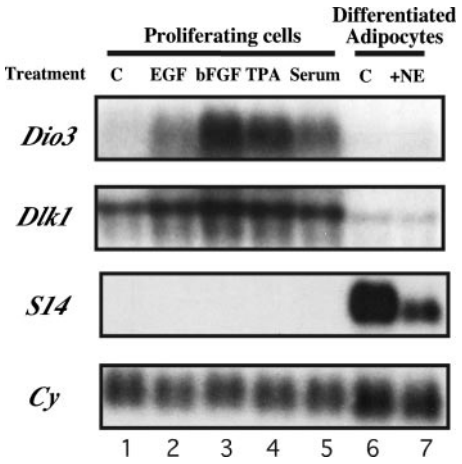

FIG. 9. Effect of serum, growth factors, phorbol esters, and NE on the gene expression pattern in proliferating precursor cells and fully differentiated brown adipocytes. Cells were cultured in special medium to promote either proliferation or differentiation (see *Materials and Methods*). Media were depleted of serum content to 2% the night before and the cells were then treated for 6 h with the agents indicated as described in the *Materials and Methods*. Doses used were 10 ng/ml of EGF and bFGF; 1 μM of tetradecanoyl phorbol acetate; 10% NCS; and 2 μM NE. Blot was autoradiographed for 2 d. C, Untreated cells used as control. Cy, Cyclophilin.

fully differentiated in each case. The similar pattern of expression of *Dio3* and *Dlk1* indicate again that *Dio3* expression tightly correlates with cells that are in an undifferentiated status. It is worth noting that even mature adipocytes can still be responsive to bFGF. Thus, in Fig. 7 we observed that *Dio3* mRNA is slightly induced by bFGF in mature brown adipocytes, and conversely, S14 mRNA decreases on bFGF treatment (Fig. 8).

Another interesting finding is the expression in brown adipocytes of one of the transcripts for *Dio3os* (Fig. 8, *bottom*), an antisense gene that partially overlaps *Dio3* and is transcribed from the opposite strand (32). Using a *Dio3os* cDNA probe, we observed that an approximately 4-kb *Dio3os* transcript is expressed in mature adipocytes, but its abundance is very low in proliferating cells (Fig. 7). Using this probe, which contains part of the *Dio3* promoter region and exon, the *Dio3* transcript (2.1 kb) is not detected, indicating that the mRNA shown is a *Dio3os* transcript. In contrast to the presence of at least three most abundant *Dio3os* transcripts in fetal mRNA (32, 46, 47), the detected *Dio3os* transcript is by far the most abundant in differentiated adipocytes.

In a similar experiment, we find that *Dio3* and *Dlk1* expression occurs concurrently in proliferating cells when stimulated with other factors such as phorbol esters (tetradecanoyl phorbol acetate) and serum (Fig. 9, *top panels*), whereas the adipocyte marker S14 is not expressed. Again mature adipocytes manifest much lower or absent expression of both *Dio3* and *Dlk1* but high expression of the lipogenic marker S14. Treatment of differentiated cells with norepinephrine, an adrenergic activator of the brown adipocyte, did not result in any change in Dkl1 or Dio3 expression, although it resulted in a decrease in S14 mRNA as described (16).

D3 activity in BAT

Because the results above may apply to BAT *in vivo*, we measured D3 activity in this tissue. Initial experiments using T_3 radiolabeled in the inner ring of the molecule showed no detectable activity. Because the D3 assay using this substrate has limited sensitivity (~75 fmol/h·mg; see *Materials and Methods*), we repeated the determinations using T_3 radiolabeled in the outer ring, which is available with higher specific activity that results in higher assay sensitivity (see *Materials and Methods*). This assay does not result in significantly different values for D3 activity (data not shown) but allows the detection of D3 at lower expression levels (see *Materials and Methods*). Thus, D3 activity in interscapular BAT was 28.5 ± 2.8 (n = 5) and 10.06 ± 1.58 (n = 4) fmol/h·mg ($P = 0.002$) in newborn and adult mice, respectively. These activities are significant and well above the sensitivity of this assay but are roughly 2 orders of magnitude lower than those found in differentiating preadipocytes in primary culture (28). These data demonstrate that some D3 is expressed *in vivo* in this tissue.

Discussion

Numerous studies using animal and cell culture models have demonstrated the importance of TH and local T_4 to T_3 conversion for BAT function and thermogenesis as well as for the proper differentiation of the brown fat cell. For this reason, our previous observation of high basal and induced expression of D3 (28), an enzyme that inactivates TH, in a cell culture model of differentiating brown adipocytes was unexpected. This finding is even more surprising because very little D3 activity is detected in BAT *in vivo*. In the present work, we provide data demonstrating that D3 expression is tightly associated with the proliferative activity of preadipocytes.

In basic culture conditions as those originally described by Nechad (23), preadipocytes in primary culture briefly proliferate, reach confluence, and begin their conversion to adipocytes. In these conditions we initially observed high level of D3 expression and then a marked decline in basal D3 activity as cells undergo differentiation. A lesser stimulation of D3 activity by growth factors was also noted in differentiating cells, suggesting that fewer cells are retaining the capability of expressing D3 or that D3 is less inducible as cells differentiate. Both results indicate that D3 is expressed to a lesser degree as adipose conversion progresses, suggesting that D3 is a marker of preadipocytes rather than mature adipocytes.

In previous works using this cell culture model, we identified a number of growth factors capable of stimulating D3 expression (28). We also studied growth factors that regulate proliferation of precursor cells and how they affect subsequent expression of brown adipocyte markers when the cells differentiate (41). These factors induce D3 expression to different extents and with different synergisms and dose dependence. Using an array of treatments to achieve a wide range of D3 expression levels, we have shown that this parameter correlates strongly with thymidine incorporation in the nuclei of proliferating preadipocytes. This correlation indicates that these factors promote both cell growth and D3 expression.

In this regard it is worth noting that serum, a treatment typically used as a positive control in experiments of cell proliferation markedly induces D3 activity in brown preadipocytes. Although T_3 is known to up-regulate D3 activity in other tissues, the T_3 that the serum contains is unlikely to have any significant effect on D3 expression because its concentration is low after dilution of the serum in the culture medium (see *Materials and Methods*). In addition, unpublished results suggest that T_3 concentration needs to be within supraphysiological values to have any significant effect on D3 activity. Even at these higher doses, its effect on D3 activity is barely 5% of that achieved by serum or growth factors. Thus, it is unlikely that serum T_3 content is having any effect *per se* on the effects observed.

The rapid induction of D3 by serum (3 h) is preceded by a transient rise in the expression of the early gene c-*jun*. This oncogenic protein is a component of the activator protein-1 factor, for which a functional response element has been identified in the D3 gene (48). In addition, we show that growth factor signaling may impair differentiation as EGF and aFGF can decrease the expression of LPL, an early marker of lipogenesis in the adipose precursor cell (49, 50). Indeed, many protocols of adipose differentiation in culture include limiting or removing serum to facilitate the final steps of fat cell differentiation. Taken together, these results indicate that D3 is associated primarily with precursor cells that are in a proliferative state. However, because of the heterogeneity of primary cultures with regard to different states of proliferation and differentiation, it is possible to transiently observe the expression of both D3 and that of molecular markers of mature adipocytes such as S14.

The association of D3 expression with proliferation of cultured brown preadipocytes raises the possibility that this enzyme may play a causal role in controlling adipose differentiation. The rapid T_3 degradation observed in these cultures in the presence of serum (and thus high D3 activity) indicates that the amount of T_3 to which the cells are exposed is sharply limited by D3 action. The scarcity of T_3 may result in a significant delay and/or impairment in the adipose differentiation process because T_3 is known to induce lipogenic and brown fat-specific markers that are critical to the adipocyte phenotype (16, 17, 19, 21). This notion is further supported by our previous observations that the action of T_3 on some of these markers is more robust in preconfluent cells or preadipocytes that are early in the differentiation process than in adipocytes cultured longer and already displaying obvious features of differentiation such as the accumulation of lipid droplets (16, 19, 21).

The tight association of D3 with the proliferation of brown fat precursor cells is most clearly observed in our experiments using different culture conditions to promote a marked difference in the proliferation/differentiation state of the cell. The results of these experiments demonstrate a much more dramatic association of D3 with proliferation. In cell culture conditions favorable for cell growth, D3 is coexpressed with *Dlk1* (preadipocyte factor 1), a gene that is a well-known inhibitor of adipose conversion in cell culture (38, 39) and that when inactivated leads to increased adiposity in mouse models (51). However, in culture conditions promoting differentiation, D3 expression inversely correlates with S14, a lipogenic marker that is highly expressed in brown and white adipose tissue (52).

Interestingly, *Dlk1* is also an imprinted gene located in the

same chromosomal domain as the *Dio3* (37). Its coexpression with *Dio3* in preadipocytes but not mature adipose cells suggests that these genes play a coordinated role in the adipocyte conversion of brown fat precursor cells. Of note, glucocorticoids can inhibit the expression of both *Dio3* and *Dlk1* in cultured preadipocytes (53, 54). Because glucocorticoids promote adipose differentiation in cell culture, the concurrent repression of both genes by these compounds again indicates the close relationship between expression pattern and proliferative status. Even more intriguing is the expression pattern of *Dio3os*, an apparently noncoding gene with unknown function that overlaps and is transcribed antisense to the *Dio3* (35). The marked increase in expression of a specific *Dio3os* transcript in differentiated cells further supports the hypothesis that coordinated gene expression at this imprinted domain may be of relevance to adipose conversion and the early stages of preadipocyte proliferation.

BAT is a well-vascularized tissue, consistent with its primary function of heat dissipation. Cytologic analysis indicates that it is composed primarily of endothelial cells and mature brown adipocytes (4, 43). Other types of cells, including precursor cells, are present in much smaller numbers (23). The low representation of precursor cells in the tissue may explain why D3 activity is modest in BAT. The significantly higher D3 activity in the newborn BAT, compared with that of the adult, is consistent with the increased growth rate and activity of BAT during perinatal life, a period in which thermogenesis is most critical. Thus, our data demonstrate that D3 function is limited but present in BAT *in vivo*. They also suggest the possibility that D3 in BAT locates mainly to these adipose progenitor cells that, upon appropriate signaling, may undergo differentiation or proliferation. Our result showing the induction of D3 activity by endothelin I in these precursor cells suggests the possibility that vascular factors may be important for keeping the balance of proliferation and differentiation in these cells and that *Dio3* may contribute to that effect.

The presence of D3 in BAT and its association with proliferative precursor cells suggest interesting potential roles for this enzyme. Because TH are critical for brown adipose differentiation and function, the presence of D3 in preadipocytes may serve a function of preventing adipose differentiation and prolonging the proliferative state of precursor cells, which may affect BAT size and cellular composition.

In summary, our data demonstrate that D3 expression in brown preadipocytes is associated with proliferation and is repressed as the cells convert into mature adipocytes. The inactivation of TH by D3 may extend the proliferative state and thus prevent or delay the differentiation of preadipocytes because TH are necessary for the expression of adipose cell markers. The coordinated expression in this cell culture model of some genes from the Dlk1/Dio3 imprinted domain suggest that they may play an important role during the processes of adipocyte proliferation and differentiation that warrants further analysis.

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